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(54) Title: PROTEASE VACCINE AGAINST HEARTWORM

(57) Abstract

The transition between the L3 and L4 larval stages of the nematode filarial parasites such as *Dirofilaria immitis* occurs after introduction into the animal host and is mediated by at least one metalloprotease and/or cysteine protease unique to the L3 or L4 larval stage. Methods to prevent and treat filarial infection are provided by vaccines comprised of the immunogenic determinants of the characteristic L3 or L4 proteases and by administration of inhibitors of these proteases.

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PROTEASE VACCINE AGAINST HEARTWORM

Technical Field

The invention relates to prevention and treatment of nematode-caused filarial disease in animal hosts, such as heartworm which occurs most commonly in dogs. Heartworm infection is caused by the nematode Dirofilaria immitis, and the treatment and prevention method of the invention can be applied specifically to this disease by employing the characteristic metallo-protease and/or cysteine protease associated with this organism.

Background Art

The heartworm infection caused by D. immitis is a widely distributed problem in dogs in most regions of the world with the exception of Africa. Current treatment is generally chemoprophylactic with agents designed to directly kill the infecting organisms. While this treatment has gained acceptance, because of the inherent toxicity of such treatment, it would be preferable to immunologically protect the host against infection, or to revise the chemoprophylactic regime to include less toxic agents. The present invention is directed to this goal.

Other nematode filarial infections are of even greater significance and involve life cycles of the infectious agent similar to those related to heartworm. For example, of more concern are the other filarids which infect humans, and more than 200 million people worldwide are estimated to have such infections. Filarids which

infect humans include Brugia malayi, Wuchereria bancrofti, and Onchocerca volvulus. These are serious infections which can cause blindness and elephantiasis in humans. At present, there is no effective vaccine available against filarial nematode infection.

As the life cycles of the infectious agents are similar in all of these diseases, heartworm infection can be used as an illustration. This life cycle can be described as follows:

Heartworm infection, specifically in dogs, generally occurs through passage of the third-stage larvae (L3) of the nematode D. immitis into the subcutaneous tissue from a mosquito vector. When these larvae are passed into the animal's tissue, their life cycle is continued by molting into a fourth larval stage (L4), which then migrates toward the heart and pulmonary arteries where the subsequent stage matures into an adult. The L3 remain at the site of inoculation by the mosquito until molting occurs. L4 emigrate through cutaneous tissue and muscle and do not molt to fifth stage for 50-70 days after infection (Grieve, R., et al., Epidem Rev (1983) 5:220-246). Adult D. immitis, which are on the order of 12-20 cm (males) and 25-31 cm (females) in length, produce, in this location, motile vermiform embryos called microfilariae, which are only 0.3 mm long and which traverse capillary beds and circulate in the vascular system. The microfilariae are ingested by mosquitoes and continue their life cycle through L3 in the mosquito vector.

The transition from L3 to L4 is thus the initial step in the infection cycle in the animal host. This transition involves a molting process, which has been studied by Abraham, D., et al., Experimental Parasitol (1990) 70:314-322, incorporated herein by reference. In this study, the morphology of the larvae

during the molt was monitored, and the roles of temperature and of albumin, which appeared to be essential for this process, were evaluated. The L3 stage contains a cuticle and epicuticle which are abandoned, and the body wall of the larva is encased in the L4 stage cuticle and epicuticle. During this molting process, an excretory-secretory (E-S) product which, among other components, contains enzymes presumably employed in the molting process is formed.

The excretory-secretory products of various tissue invasive helminths have been studied. Proteases have been found in a number of them. Both Schistosoma mansoni and Schistosomatium douthitti produce elastases capable of degrading skin (McKerrow, J.H., et al., Experimental Parasitol (1982) 53:249; McKerrow, J.H., et al., J Biol Chem (1985) 231:47; Amiri, P., et al., Mol Biochem Parasitol (1988) 28:113). Fasciola hepatica also releases a number of proteolytic enzymes (Dalton, J.P., et al., Mol Biochem Parasitol (1989) 35:161). The adult hookworm Ancylostoma caninum releases a histolytic protease and a protease that acts as an anticlotting agent (Hotez, P.J., et al., J Biol Chem (1985) 260:7343). Toxocara canis larvae secrete proteases which degrade components of extracellular matrix (Robertson, B.D., et al., Experimental Parasitol (1989) 69:30).

A number of filarial nematodes also have been shown to produce proteases that act on extracellular matrix components, including Onchocerca cervipedis, O. cervicalis, and Brugia malayi (Lackey, A., et al., Experimental Parasitol (1989) 68:176; Petralanda, I., et al., Mol Biochem Parasitol (1986) 19:51). The protease activity includes collagenase in the case of Brugia malayi, O. cervicalis and O. cervipedis. A collagenase and a leucine aminopeptidase have been found in the molting process of Haemonchus contortus by Rogers, W.P.,

J Parasitol (1982) 12:495, and Gamble, H.R., et al., Mol Biochem Parasitol (1989) 33:49.

With respect to D. immitis, protease activity has been detected in soluble extracts of D. immitis adults by Maki, J., et al., J Helminthol (1986) 60:31-37, and in extracts of microfilariae by Tomashiro, W.K., et al., J Parasitol (1987) 73:149-154. However, the soluble extracts of D. immitis L3 and L4 and the excretory-secretory (E-S) products of these larval stages have not previously been studied.

It is also known that collagens form the major structural components of nematode cuticle by virtue of studies conducted on Caenorhabditis elegans, B. malayi, and B. pahangi.

Disclosure of the Invention

The invention is directed to prevention and treatment of filarial nematode infection in animal hosts and to purified and isolated forms of the proteases associated with the L3 and L4 larval stages of the parasites that cause these infections. One of these nematodes is Dirofilaria immitis, which causes heartworm in dogs. Other diseases of importance are caused by nematodes such as those listed above. The invention provides an approach to the eradication of conditions caused in animals by filarial nematodes, and provides materials useful in these and in in vitro contexts.

Accordingly, in one aspect, the invention is directed to a method to protect animal subjects, including humans, against filarial nematode infection, which method comprises administering to the subject an effective amount of a metalloprotease and/or cysteine protease characteristic of transition from the L3-L4 stage of the relevant filarial nematode effective to immunologically protect the subject against infection.

The characteristic metalloprotease(s) may be found in the L3 or L4 excretory-secretory material or in L3 or L4 lysates. The cysteine protease is found in L3 and L4 lysates.

In another aspect, the invention is directed to the treatment of nematode filarial infection in animal subjects, including humans, which method comprises administering to that subject an effective amount of a metalloprotease inhibitor and/or cysteine protease inhibitor.

In other aspects, the invention is directed to antibodies immunospecific for filarial L3 or L4 excretory-secretory products or L3 or L4 lysate metalloprotease(s) or to L3 or L4 lysate cysteine protease(s) and to pharmaceutical compositions and vaccines containing them.

In still another aspect, the invention is directed to the L3/L4-associated metalloproteases and cysteine proteases of filarial parasites in isolated and purified form. These purified proteases are additionally useful to assay for the presence or absence of antibodies in the diagnosis of affected individuals and to regulate the growth of cell cultures in vitro, as well as in other therapeutic applications.

Brief Description of the Drawings

Figure 1 shows the elution pattern of protease activity from L3/L4 E-S.

Figure 2 shows the elution pattern of protease activity from L4 lysate.

Modes of Carrying Out the Invention

As used herein, "metalloprotease" of L3 and L4 excretory-secretory preparation (L3 and L4 E-S) or of L3 or L4 lysates refers to metalloprotease enzymes characteristic of the excretory-secretory products obtained during the molting of the L3 larval stage into L4 for filarial infective nematodes; or of whole worm lysates of the L3 or L4 larval stage. At least one "cysteine protease" is also found in L3 and L4 lysates. While the E-S and lysate preparations from D. immitis are exemplified below, similar E-S or lysate preparations can be obtained from various other filarial parasites such as those set forth in the Background section above, and including, specifically, for example, B. malayi, W. bancrofti, O. volvulus, Dipetalonema perstans, D. streptocerca, Mansonella ozzardi, and Loa loa.

Preparation of Larval Cultures

The parasites can be cultured in vitro under suitable conditions to provide a source for the E-S preparation or for the L3 or L4 lysates. For example, D. immitis can be cultured as described by Abraham, D., et al., J Parasitol (1987) 73:377-383. Briefly, the mosquito Aedes aegypti Liverpool (black-eyed strain) are infected with D. immitis by feeding on microfilaremic blood obtained from a single experimentally infected dog. Fifteen days after feeding, the mosquitos are anesthetized, surface sterilized and placed on screens in funnels filled with a 1:1 mixture of NCTC-135 and Iscove's modified Dulbecco medium (Sigma) containing 2.5 μg/ml amphotericin-B; 100 μg/ml gentamicin; 50 μg/ml sulfadiazone; and 10 μg/ml trimethoprim. The larvae are collected from funnels 90 minutes postincubation.

The cultures are maintained at a concentration of ten L3 organisms per ml of medium in 5% CO_2 and

saturated humidity. The larvae (L3) are cultured at 37° in the foregoing medium, supplemented with 20% fetal calf serum for 1-8 days.

Alternatively, and preferably, 10 days after feeding, the mosquitos are anesthetized and the worms are recovered by dissecting the heads and allowing the worms to emerge into medium with 20% Seru-max (Sigma) to induce molting. After 48 hr, the worms are recovered, washed 5 times in medium which does not contain Seru-max, and recultured therein.

Preparation of L3 and L4 E-S

L3 ES is collected between 48 and 96 hours of culture on Seru-max free medium. L4 ES is collected between 96 and 144 hours in indentical culture conditions. Medium containing ES is collected and filtered through a 0.45 μm filter. The ES is concentrated and the buffer is exchanged into pH 7.2 PBS using ultrafiltration and 10 kd exclusion limit to obtain the fraction of >10 kd MW.

Preparation of L3 and L4 Lysates

Larval soluble extracts are prepared from L3 collected on day 2, just after the wash but prior to the molt, and L4 are collected on day 6 in serum-free culture. Pellets of 10,000 worms in PBS are disrupted by ten 10-sec high frequency pulses using a tissue sonicator. Sonicated worms are centrifuged for 5 min at 12,000 x g, and the supernatant collected.

Determination of E-S and Lysate Components

Protein concentration for both E-S and whole worm soluble extracts may be estimated using a Micro BCA kit (Pierce Chemical Co., Rockford, IL). All samples are maintained at -20° C prior to further analysis.

By "metalloprotease" of the L3 and L4 E-S preparation or of L3 or L4 lysates is meant a protease enzyme which is found in the excretory-secretory product of third or fourth stage larvae or in L3 or L4 lysates of a filarial nematode parasite as ascertained by activity against the synthetic substrate h-phenylalanine-AMC (h-F-AMC, defined below) and which is inhibited by metallo-protease inhibitors such as 1,10-phenanthroline and EDTA. Metalloprotease activity has been reported in E-S products of third stage larvae of certain species, including B. malayi, O. cervicalis, and O. cervipedis as set forth above. The activity is also present in L3 and L4 lysates.

The invention also relates to "cysteine protease(s)" from L3 or L4 lysates, which lysates may be prepared as described above. The cysteine proteases of the invention are characterized by ability to hydrolyze Z-valine-leucine-arginine-AMC (Z-VLR-AMC, defined below) and this activity is inhibited by E64. Again, D. immitis is used for illustration below, but other filarial nematodes may be used.

Both of these enzymes may be obtained in purified and isolated form using chromatographic methods with use of the appropriate substrate assay to monitor elution fractions as further described below.

Because of the practical difficulties in obtaining sufficient quantities of the metalloprotease of L3 and L4 E-S preparations or the metalloprotease or cysteine protease from L3 or L4 lysates to provide material for vaccines, alternative methods of production are preferred when large quantities are desired. Specifically, for the full-length metalloprotease or cysteine protease, recombinant production is the most practical approach; for immunogenic subunits which are capable of eliciting antibodies that neutralize the

metalloprotease or cysteine protease activity, ordinary solid phase peptide synthesis may be preferred. However, even in this instance, it may be desirable to utilize recombinant production to obtain tandem repeats of the immunogenic subunit. Production of tandem repeats may enhance the immunogenicity of the material. In addition, the subunit vaccines may be recombinantly produced as fusion proteins to an immunogenicity-conferring sequence.

Recombinant Production

The recombinant sequences necessary for production of the relevant metalloprotease or cysteine protease are obtained in a process analogous to that described by Sakanari, J.A., et al., Proc Natl Acad Sci (1989) 86:4863. In this process, the gene encoding the metalloprotease or cysteine protease is isolated from cDNA prepared from total mRNA of the L3 or L4 stage of the parasite using oligonucleotide primers and the polymerase chain reaction (PCR) and suitable probes.

D. immitis genomic or cDNA is used as a source for protease-encoding genes. For isolation of the metalloprotease gene, primers are designed based on consensus sequences in the bacterial metalloprotease thermolysin, and members of the human metalloprotease family which include stromelysin, stromelysin II, and Pump-I. These highly homologous genes are all metalloproteases, and the cDNAs containing these sequences have been disclosed (Muller, D., et al., Biochem J (1988) 253:187-192 and Quantin, B., et al., Biochemistry (1989) 28:5327-5334). Primers can be designed based on the conserved regions, including the active site. PCR amplification is conducted as described by Sakanari et al. (supra), and reaction products are loaded on agarose/NuSieve (FMC). An additional probe designed based on the above-mentioned published sequences

is used to identify the relevant amplified gene products on Southern blots. The fragments are cut out of the gel, extracted with "glass milk" (Geneclean) and ligated into Bluescript (Stratagene) to obtain sequences of both coding and anticoding strands, using the dideoxy method of Sanger with Sequenase (USB) and the SK and KS primers for sequencing in both directions. The gene fragments obtained are then used as probes to screen a cDNA library. They are labeled with ^{32}P using standard random priming methods.

For preparation of analogous probes for the cysteine protease genes, primers are designed based on the sequences disclosed in Eakin, A.E. et al., Mol Biochem Parasitol (1990) 39:1-8. Otherwise, the retrieval of probes from genomic DNA can be conducted as above.

The cDNA library is constructed from messenger RNA isolated from third stage larvae which have been in culture for 48-72 hours. The mRNA is isolated by the single step acid guanidinium thiocyanate/phenol/chloroform extraction method of Chomczynski, P. and Sacchi, N., Anal Biochem (1987) 162:156-159. The RNA is passed over an oligo-dT cellulose column and the poly-A RNA is eluted using standard procedures. cDNA is prepared from the mRNA using standard procedures such as those of Gubler, U. and Hoffman, B.J., Gene (1983) 25:263. The cDNA is treated by methylation of internal EcoRI sites, and phosphorylated EcoRI linkers are added to the ends of the cDNA and treated again with phosphatase. The treated cDNA contain linkers digested with EcoRI to generate cohesive cloning ends for insertion into λ-gt11 arms (Stratagene, San Diego, CA) and packaged using Gigapack (Stratagene). Standard methods are used to titer and plate the library for screening.

The library can be screened either using the probes obtained as described above, heterologous probes, or the expression products can be screened using antibodies prepared against the proteases obtained from the E-S product or lysates. Selected clones are plaque purified, and the isolated coding sequences are used to produce the recombinant protease.

The cloned DNA can be used directly in expression vectors, or DNA can be synthesized using standard solid phase techniques to obtain any embodiment of the coding sequence to supply all or a portion of the gene.

For example, a DNA coding sequence for the protease can be prepared synthetically from overlapping oligonucleotides whose sequence contains codons for the amino acid sequence encoded in the native gene. Such oligonucleotides are prepared by standard methods and assembled into a complete or partial coding sequence. See, e.g., Edge, Nature (1981) 292:756; Nambair et al., Science (1984) 223:1299; Jay et al., J Biol Chem (1984) 259:6311.

Thus, a DNA molecule containing the coding sequence for the filarial nematode metalloprotease or cysteine protease can be cloned in any suitable vector and thereby maintained in a composition substantially free of vectors that do not contain the coding sequence for the protease (e.g., other library clones). Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Examples of recombinant DNA vectors for cloning and the host cells which they transform include bacteriophage λ (E. coli), pBR322 (E. coli), pACYC177 (E. coli), pKT230 (gram-negative bacteria), pBG1106 (gram-negative bacteria), pLAFR1 (gram-negative bacteria), pME290 (non-E. coli gram-negative bacteria),

pHV14 (E. coli and Bacillus subtilis), pBD9 (Bacillus), pIJ61 (Streptomyces), pUC6 (Streptomyces), actinophage φC31 (Streptomyces), YIp5 (yeast), YCp19 (yeast), and bovine papilloma virus (mammalian cells).

For expression, the coding sequence of the protease gene is placed under the control of a promoter, ribosome binding site (for bacterial expression) and, optionally, an operator (collectively referred to herein as "control" sequences) so that the protease-encoding sequence is transcribed into RNA in the host cell transformed by the vector. The coding sequence may or may not contain a signal peptide or leader sequence. In bacteria, the protease is preferably produced by the expression of a coding sequence which does not contain any native signal peptide, or by expression of a coding sequence containing the leader sequence in a eucaryotic system when post-translational processing removes the leader sequence. The protease can also be expressed in the form of a fusion protein, wherein a heterologous amino acid sequence is expressed at the N- or C-terminus. See, e.g., U.S. Patent Nos. 4,431,739; 4,425,437.

The recombinant vector is constructed so that the protease-encoding sequence is located in the vector with the appropriate control sequences, the positioning and orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the control of the control sequences (i.e., by RNA polymerase which attaches to the DNA molecule at the control sequences). The control sequences may be ligated to the coding sequence prior to insertion into a vector, such as the cloning vectors described above. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequence and an appropriate restriction site downstream from control sequences. For

expression of the protease-encoding sequence in other than nematodes, the control sequences will be heterologous to the coding sequence. If the host cell is a procaryote, it is also necessary that the coding sequence be free of introns; e.g., cDNA. If the selected host cell is a nematode cell, the control sequences can be heterologous or homologous to the protease-encoding sequence, and the coding sequence can be genomic DNA containing introns or cDNA. Either genomic or cDNA coding sequences may be also expressed in yeast.

A number of procaryotic expression vectors are known in the art. See, e.g., U.S. Patent Nos. 4,440,859; 4,436,815; 4,431,740; 4,431,739; 4,428,941; 4,425,437; 4,418,149; 4,411,994; 4,366,246; 4,342,832. Preferred expression vectors, however, are those for use in eucaryotic systems. Yeast expression vectors are known in the art. See, e.g., U.S. Patent Nos. 4,446,235; 4,443,539; 4,430,428. See also European Patent Specifications 103,409; 100,561; 96,491. The recombinant protease can be produced by growing host cells transformed by the expression vector described above under conditions whereby the protease is produced. Human collagenase cDNA has been cloned and expressed in active form in eucaryotic cells (Muller, D., et al., Biochem J (1988) 253:187-192). The protease is then isolated from the host cells and purified. If the expression system secretes the protease into growth media, the desired protein can be purified directly from cell-free media. If the protease is not secreted, it is isolated from cell lysates. The selection of the appropriate growth conditions and recovery methods are within the skill of the art; purifications similar to those exemplified below can be used.

Antibody Production

Either native or recombinant proteases of the invention can be used to produce antibodies, both polyclonal and monoclonal. If polyclonal antibodies are desired, the purified protease is used to immunize a selected mammal (e.g., mouse, rabbit, goat, horse, etc.) and serum from the immunized animal later collected and treated according to known procedures. Compositions containing polyclonal antibodies to a variety of antigens in addition to the relevant protease can be made substantially free of antibodies which are not protease antibodies by passing the composition through a column to which the desired protease has been bound. After washing, polyclonal antibodies are eluted from the column. Monoclonal antibodies can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal, antibody-producing cell lines can also be created by techniques other than fusion, such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See, e.g., Schreier, M., et al., HYBRIDOMA TECHNIQUES (1980); Hammerling et al., MONOCLONAL ANTIBODIES AND T-CELL HYBRIDOMAS (1981); Kennett et al., MONOCLONAL ANTIBODIES (1980).

By employing the metalloprotease or cysteine protease (native or synthetic) as an antigen in the immunization of the source of the B-cells immortalized for the production of monoclonal antibodies, a panel of monoclonal antibodies recognizing epitopes at different sites on the protease can be obtained. Antibodies which recognize an epitope in the active site binding region of the protease can be readily identified in competition assays between antibodies and enzyme substrate. Artificial substrates such as Z-VLR-AMC (cysteine

protease) or h-F-AMC (metalloprotease) can also be used. Such antibodies have therapeutic potential if they are able to block the binding of protease to its substrate in vivo. Antibodies which recognize a site on the protease are also useful, for example, in the purification of the desired protease protein from cell lysates or fermentation media, and in its characterization. In general, as is known in the art, the protease antibody is fixed (immobilized) to a solid support, such as a column or latex beads, contacted with a solution containing the protease, and separated from the solution. The protease, bound to the immobilized antibodies, is then eluted.

Isolation and Purification of the L3 and L4 Proteases

As stated above, the cysteine protease characteristic of the L3 and L4 lysates and the metalloprotease characteristic of these lysates, as well as the L3/L4 E-S, can be obtained in isolated and purified form either using the appropriate larval stage of the desired parasitic nematode as starting material, using recombinant production in cell culture and isolating the protease resulting from the cysteine protease or metalloprotease gene expression, or by synthesizing subunits of these proteins using standard peptide synthesis techniques. The nature of the purification method will depend on the origin of the protease or peptide.

When isolated from native sources, the lysate or E-S material is subjected to chromatographic techniques, typically chromatography using affinity chromatography (e.g., affinity chromatography using antibodies prepared with respect to the protease as affinity ligands), ion-exchange chromatography, sizing columns, reverse-phase columns, and the like. Optimization of the purification procedure is within the

skill of the art, as the fractions eluted from the columns can be assayed using activity determination with a fluorometric substrate characteristic of the metalloprotease or cysteine protease. For the metalloprotease of D. immitis, h-F-AMC is a convenient substrate; for the cysteine protease of this worm, Z-VLR-AMC is appropriately used. The specificity and nature of the protease can be verified by supplementing the assay with various inhibitors known to characterize metalloproteases or cysteine proteases. Modified forms of these substrates may be appropriate for the metalloproteases or cysteine proteases of other species of filarial nematodes; the appropriate substrate can be ascertained by the conduct of preliminary assays on the crude extracts, as exemplified herein for the D. immitis species.

If the protease is produced recombinantly, similar techniques can be used, although the starting material generally contains the protease in a more highly concentrated form. Further modification of the purification procedure is appropriate for isolation of the peptides prepared by solid-phase synthesis, since the nature of the contaminants is different. Generally, dialysis or other size-separation methods are appropriate.

The purified and isolated forms of the cysteine and metalloproteases of the various filarial nematode species can be used in the production of antibodies (which antibodies, in turn, are useful in immunoassays and separation techniques), as reagents in immunoassay procedures for the presence or absence of antibodies, and in the regulation of cell culture in vitro by controlling extracellular matrix formation or status.

Use of Purified and Isolated Proteases in Diagnosis

The purified and isolated proteases are useful in diagnostic immunoassays for the presence or absence of antibodies with respect to filarial nematode species. These assays can be used to assess the disease state of a host organism or to assay titers in immunization protocols. The assays are conducted in standard immunological format, including RIA, ELISA, and fluorescence-labeled assays. The assays can be conducted in either a direct or a competitive format and rely on separations by virtue of binding to solid support or by virtue of precipitation of immunological complexes. A large number of protocols suitable for the conduct of immunoassays is well known in the art.

Use of Purified and Isolated Proteases in Vaccines

The proteases of the invention are useful as vaccines in immunizing host organisms to protect them against infection by the corresponding filarial nematode. The proteases are administered in standard pharmaceutical formulations systemically, and typically by injection. Injection may be intravenous, intramuscular, peritoneal, or other parenteral. Suitable vehicles for injection include physiological saline, Hank's solution, Ringer's solution and the like, with or without the presence of adjuvants, according to the immunization protocol. Generally, the vaccine is administered at a dosage level sufficient to raise antibody titers to provide effective scavenging of the proteases required for molting from the L3 to the L4 stage in the filarial infective agent.

Treatment of Infection with Inhibitors

As the proteases of the invention are needed for the progression of the parasitic nematode life cycle, administration of inhibitors of these enzymes to infected hosts in suitable dosages inhibits or arrests the course of the infection. The inhibitors are formulated in suitable pharmaceutical compositions such as those described in Remington's Pharmaceutical Sciences, latest edition, Mack Publishing Co., Easton, PA. Administration is preferably by oral formulation, although injection or transdermal or transmucosal routes can also be used.

The following examples are intended to illustrate, but not to limit the invention.

Example 1

Demonstration of Protease Activity in D. Immitis

D. immitis were cultured at a concentration of 100 larvae per ml in a 1:1 mixture of NCTC-135 and Iscove's modified Dulbecco medium (Sigma) containing antibiotics (NI) on a model of extracellular matrix (ECM) secreted by rat vascular smooth muscle cells and labelled with tritiated proline. Every 8 hrs a 50 μl sample was collected and the amount of tritium released from the matrix was counted on a scintillation counter. The counts per minute of tritium released from the ECM for the L3 stage increased slowly from 1 x 10^4 cpm after 8 hours to about 2 x 10^4 cpm after 56 hours, when L3 molting occurs. A large incremental release of tritium (indicating degradation of the matrix) occurs at the time of L3 molting; cpm increase to about 6 x 10^4 cpm after 64 hrs and to over 8 x 10^4 cpm after 72 hrs. The breakdown of matrix mediated by L4 tracked that by L3 until the 56 hour L3 molt event; cpm for L4 continued to increase only slowly after this (to <4 x 10^4 cpm after 72 hrs). In

total, after 72 hours the L3 culture degraded 20% of the total ECM, and the L4 culture degraded 13%.

The components of the ECM which were degraded were evaluated by sequential enzyme digests of the remaining ECM as described previously (McKerrow, J.H., et al., Lab Invest (1983) 49:195-200.

The results are shown in Table 1. Collagen is shown to be the major component of the ECM degraded by both L3 and L4 lysates. However, L3 lysates degrade nearly twice as much collagen as lysate from L4.

Table 1
Percent Degradation of ECM Constituent Proteins
(100 larvae lysate/ml)

	L3	L4
Glycoproteins	22	20
Elastin	10	8
Collagen	61	38

In these experiments, controls for nonparasite-derived degradation of ECM constituted either NI alone, mosquito media, or C. elegans. Mosquito media were prepared from noninfected mosquito heads processed as if they contained worms. C. elegans adults and larvae were recovered from NGM agarose plates seeded with E. coli strain OP50, placed in M9 media at the same concentration as the D. immitis larvae and incubated at 26°C. Mosquito media were used as a control to assure that mosquito-derived proteases were not responsible for any of the degradation observed. C. elegans, a free-living nematode, was used as a comparison to tissue-invasive nematodes and as a control to insure motility alone did not cause release of label.

Lysates from L3 and L4 were prepared by sonication of the larvae in PBS on ice using 10 x 10 sec high frequency pulses. Lysates containing 10 μg protein per reaction were tested against artificial substrates consisting of amino acids linked to a fluorogenic compound, 7-amido-4-methylcoumarin (AMC) (Bachem). Some substrates were protected against exopeptidase activity by a benzyloxycarbonyl group, abbreviated Z; the substrates that are not protected are indicated by a preceding "h". These substrates are Z-Val-Leu-Arg-AMC, h-Phe-AMC, Z-Phe-Arg-AMC, and Z-Arg-Arg-AMC (abbreviated Z-VLR-AMC, h-F-AMC, Z-FR-AMC, and Z-RR-AMC respectively). The lysate was incubated with each substrate for 3 hrs, and the amount of AMC hydrolyzed was measured fluorimetrically. Cleavage of AMC was measured using an LS-2 spectrofluorometer (Perkin Elmer) with 380 nm excitation wavelength and emission detection at 460 nm. Initial substrate screening reactions consisted of 10 μl substrate (at 5 mM in DMSO), 980 μl PBS, pH 7.2, and 10 μl combined L3 and L4 soluble extracts. A number of additional substrates, found to be uncleaved, were also tested: Z-GPLGP-AMC, Z-GPR-AMC, Z-ARR-AMC, Z-AR-AMC, Z-R-AMC and h-L-AMC. However, further work was conducted with the four substrates listed above. Two mM dithiothreitol (DTT) was found to enhance cleavage of Z-VLR-AMC twofold, but to inhibit h-F-AMC hydrolysis as shown in Table 2. Thus, Z-VLR AMC is shown to be a substrate for this cysteine protease, h-F-AMC is a substrate for the metalloprotease.

Table 2

Effect of 2 mM DTT on Hydrolysis of
z-VLR-AMC and h-F-AMC by L3 Soluble Extract

Substrate	nMoles AMC released/hr	
	+DTT	-DTT
h-Phe-AMC	99.0 (16.7)	326.4 (25.0)
z-Val-Leu-Arg-AMC	54.6 (1.7)	32.6 (2.6)

Data represent the means of duplicate samples with ranges indicated in parentheses.

Thereafter, in the determinations, the PBS contained 2 mM DTT except when h-F-AMC was used.

Further, reaction mixtures consisted of 10 μl 5 mM substrate, 10 μl larval soluble extract or E-S at protein concentration of 1 μg/ml and 980 μl of PBS, pH 7.2. After incubation of the lysate or E-S with substrate at 37°C for a specified length of time, the hydrolyzed AMC was measured on a Perkin Elmer LS-2 filter fluorometer with excitation and emission wavelengths as set forth above. For h-F-AMC, the L3 lysate releases about 20 μmol of AMC after the 3 hr incubation while the L4 lysate releases slightly less than 10 μmol. Z-FR-AMC and Z-RR-AMC are not effective substrates for either lysate; approximately 5 μmol of AMC are released from Z-VLR-AMC by either extract.

The excretory-secretory materials were also tested for activity on these substrates. Using 2 μg of protein per reaction, the L3 E-S composition released about 9 μmol AMC from h-F-AMC per reaction mixture after 3 hr whereas the L4 E-S composition released only about 2

μmol. No AMC was released from the Z-VLR-AMC, Z-FR-AMC or Z-RR-AMC substrates.

The effect of inhibitors was also tested with respect to the synthetic substrates Z-VLR-AMC and h-F-AMC using the lysates of L3 and L4 prepared as above. h-F-AMC was shown to be a substrate for this metalloprotease; Z-VLR-AMC was shown to be a substrate for this cysteine protease (DTT enhances cysteine protease activity; oxidizing conditions inhibit it) (see Table 2). Ten μl (10 μg total protein) of L3 or L4 lysate was mixed with 10 μl 50 μM synthetic peptide substrate, 960 μl PBS buffer, pH 7.2 containing 2 mM DTT when Z-VLR-AMC was used and without DTT when h-F-AMC was used. Twenty μl of the test inhibitors were added to the reaction mixtures, the final concentrations of the inhibitors were as follows: PMSF, 2 mM; 1,10-phenanthroline, 10 mM; NEM, 2 mM; E-64, 10 mM; Bestatin, 1 mM; Cystatin, 4 μM; and EDTA, 2 mM. Inhibition was calculated as the percent activity remaining as compared to control in absence of the inhibitor. The results are shown in Table 3 (L3) and Table 4 (L4).

Table 3

L3 Lysate % Control Activity Remaining

	Substrate	
	Z-VLR-AMC	h-F-AMC
PMSF	63	11
NEM	54	22
E64	12	100
1,10-Phe	31	8
Bestatin	62	100
Cystatin	60	100
EDTA	21	5

Table 4

L4 Lysate % Control Activity Remaining

	Substrate	
	Z-VLR-AMC	h-F-AMC
PMSF	92	20
NEM	20	23
E64	15	78
1,10-Phe	24	5
Bestatin	80	88
Cystatin	60	97
EDTA	18	4

E64, a potent cysteine protease inhibitor, had essentially no effect on the metalloprotease substrate h-F-AMC; however, E64 was the most effective inhibitor for the cysteine protease substrate, Z-VLR-AMC.

The activity of L4 lysates with respect to the various fluorogenic synthetic substrates was also tested in the presence and absence of DTT. DTT seemed to enhance the activity with respect to Z-VLR-AMC, Z-FR-AMC and Z-RR-AMC. DTT is known to enhance the activity of cysteine proteases and to inhibit metalloproteases.

The effect of the same inhibitors was tested essentially as described above with respect to the hydrolysis of h-F-AMC by L3 E-S. The reactions contain 10 µl L3 E-S, i.e., 10 µg total protein, 10 µl of the h-F-AMC to give 50 µM final concentration, 960 µl PBS pH 7.2 and 20 µl inhibitor to give final concentrations as for the lysates above. The inhibition pattern, as shown in Table 5, is similar to that shown by the lysates.

Table 5

E-S from L3: % of Control Activity Remaining

	Substrate h-F-AMC
PMSF	22
NEM	26
E64	79
1,10-Phe	5
Bestatin	80
Cystatin	98
EDTA	5

Taken together, these data show that the lysates and E-S preparations contain metalloprotease, but only the lysates contain significant amounts of cysteine protease activity.

Example 2

Preparation of Proteases from D. Immitis

The L3 or L4 lysates or the L3/L4 E-S prepared as described above were subjected to size exclusion chromatography.

The L3/L4 E-S of 8,000 larvae, collected from 48 to 144 hours, was concentrated to 75 µl in 0.05 M Tris/HCl, pH 6.8, 0.15 M NaCl, and injected for size exclusion chromatography into a TSK 3,000 SW 7.5 x 300 mm column with a 7.5 x 75 mm guard column (Beckman, Fullerton, CA) attached to Beckman Model 338 HPLC. The mobile phase used the same buffer, the flow rate was 0.5 ml/min and the detector was set at 220 nm. One minute fractions were collected starting at 12 minutes. The column was calibrated using gel filtration molecular weight markers (NW-GF-200) (Sigma).

The L3/L4 E-S which had been metabolically labeled with S35 methionine and cysteine was collected

and chromatographed under the conditions described above and the fractions were subjected to reducing SDS-PAGE using standard techniques. Figure 1 shows the chromatogram obtained when h-F-AMC was used as a substrate to assay activity of the fractions--20 μl of each fraction was incubated with 5 mM h-F-AMC in 970 ml PBS, pH 7.2, for 1 hour. Peak enzyme activity was in fraction 10 which corresponded to a molecular weight of approximately 49-58 kd. SDS-PAGE analysis of fraction 10 gave three prominent bands at 58, 30 and 22 kd and three minor bands at 28, 26 and 19 kd under denaturing and reducing conditions.

Similar separations were run using lysates prepared from 6,000 L4 worms collected after 144 hours. The fractions were assayed using both Z-VLR-AMC and h-F-AMC as substrates. The results are shown in Figure 2. The h-F-AMC activity (metalloprotease) eluted at a position corresponding to 49-54 kd; the Z-VLR-AMC activity eluted at a position corresponding to 31-34 kd.

Claims

1. A veterinary or pharmaceutical composition for immunization of an animal host against filarial nematode infection, which composition comprises an amount of at least one protease in purified and isolated form effective to immunize said animal host and which protease is obtainable by isolation from L3 or L4 lysate or from L3/L4 excretory-secretory material of said filarial nematode, or an immunogenic subunit thereof.

2. A veterinary or pharmaceutical composition useful in treating or ameliorating the symptoms of filarial nematode infection in an animal host, which composition comprises an effective amount of an inhibitor of at least one protease which protease is obtainable by isolation from L3 or L4 lysate or from L3/L4 excretory-secretory material of said filarial nematode.

3. The composition of claim 1 or 2 wherein said protease is a metalloprotease or a cysteine protease.

4. The composition of claim 1 or 2 wherein the nematode is D. immitis filarial nematode.

5. A method to immunize an animal host, susceptible to infection by a filarial nematode, against said infection, which method comprises:

administering to a host in need of such immunization an amount effective to immunize said host of at least one protease in isolated and purified form, which protease is obtainable by isolation from L3 or L4 lysate or from L3/L4 excretory-secretory material of said filarial nematode, or an immunogenic subunit thereof.

6. A method to treat or ameliorate filarial nematode infection in an animal host, which method comprises administering to said host an effective amount of an inhibitor of at least one protease which protease is obtainable by isolation from L3 or L4 lysate or from L3/L4 excretory-secretory material of said filarial nematode.

7. The method of claim 5 or 6 wherein said protease is a metalloprotease or a cysteine protease.

8. The method of claim 5 or 6 wherein the nematode is D. immitis filarial nematode.

9. Antibodies specifically immunoreactive with at least one protease which is obtainable by isolation from L3 or L4 lysate or from L3/L4 excretory-secretory material of a D. immitis filarial nematode, wherein said protease is a cysteine protease or a metalloprotease.

10. A protease obtainable by isolation from the L3 or L4 lysate or from L3/L4 excretory-secretory material of a D. immitis infective filarial nematode in purified and isolated form, wherein said protease is a metalloprotease or a cysteine protease.

11. A method to purify a protease from an L3 or L4 lysate or from L3/L4 excretory-secretory material of a filarial nematode, which method comprises subjecting said lysate or excretory-secretory material to a column chromatographic procedure and assaying fractions eluted from said column for proteolytic activity on a synthetic substrate characteristic of said protease.

12. The method of claim 11 wherein said protease is a cysteine protease and the substrate is Z-VLR-AMC, or wherein said protease is a metalloprotease and the substrate is h-F-AMC.

13. A peptide which consists essentially of an immunogenic subunit of the purified protease of claim 10.

14. A DNA in purified and isolated form that encodes the protease of claim 10, or the complement thereof.

15. An expression system capable, when transformed into a recombinant host cell, of expressing a DNA encoding the protease of claim 10.

16. Recombinant host cells transformed with the expression system of claim 15.

17. A method to prepare a protease enzyme which method comprises culturing the cells of claim 15 under conditions that effect the expression of said coding sequence and recovering the protease from the cell culture.

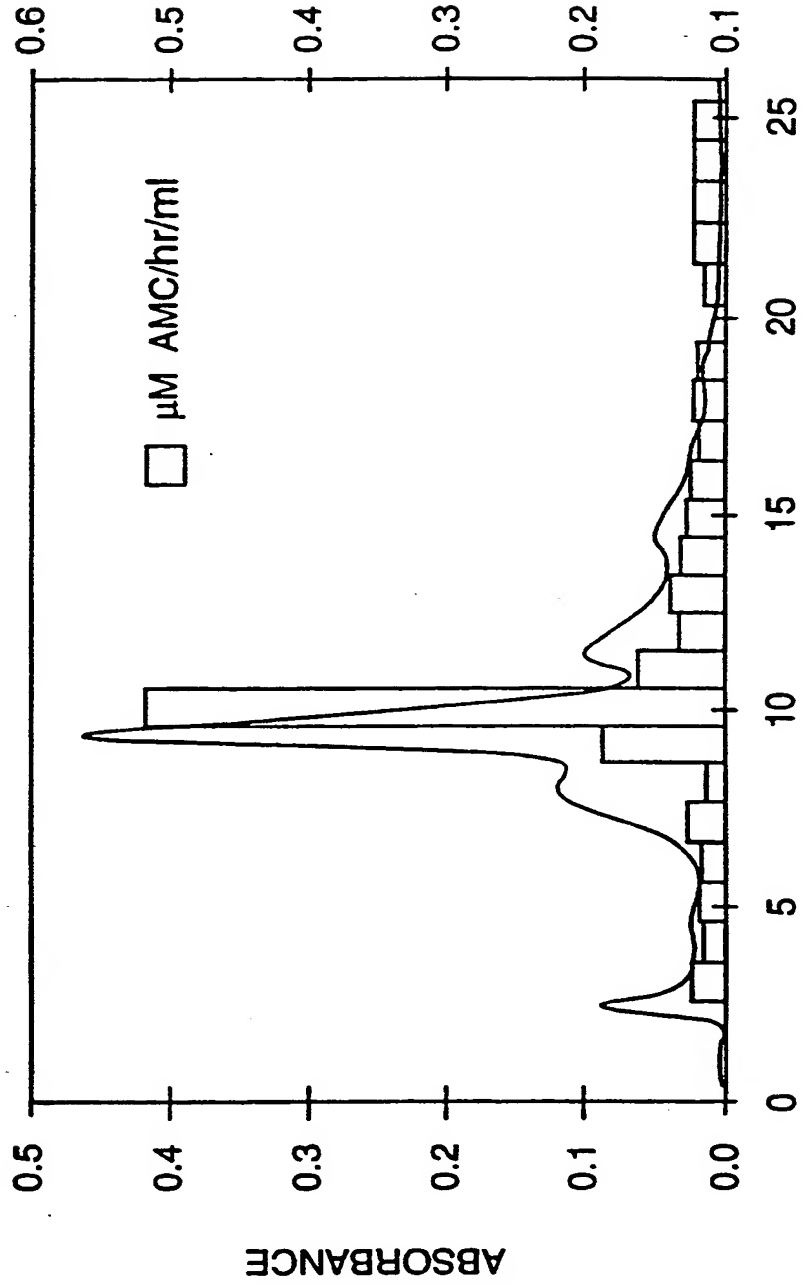

FIG. 1

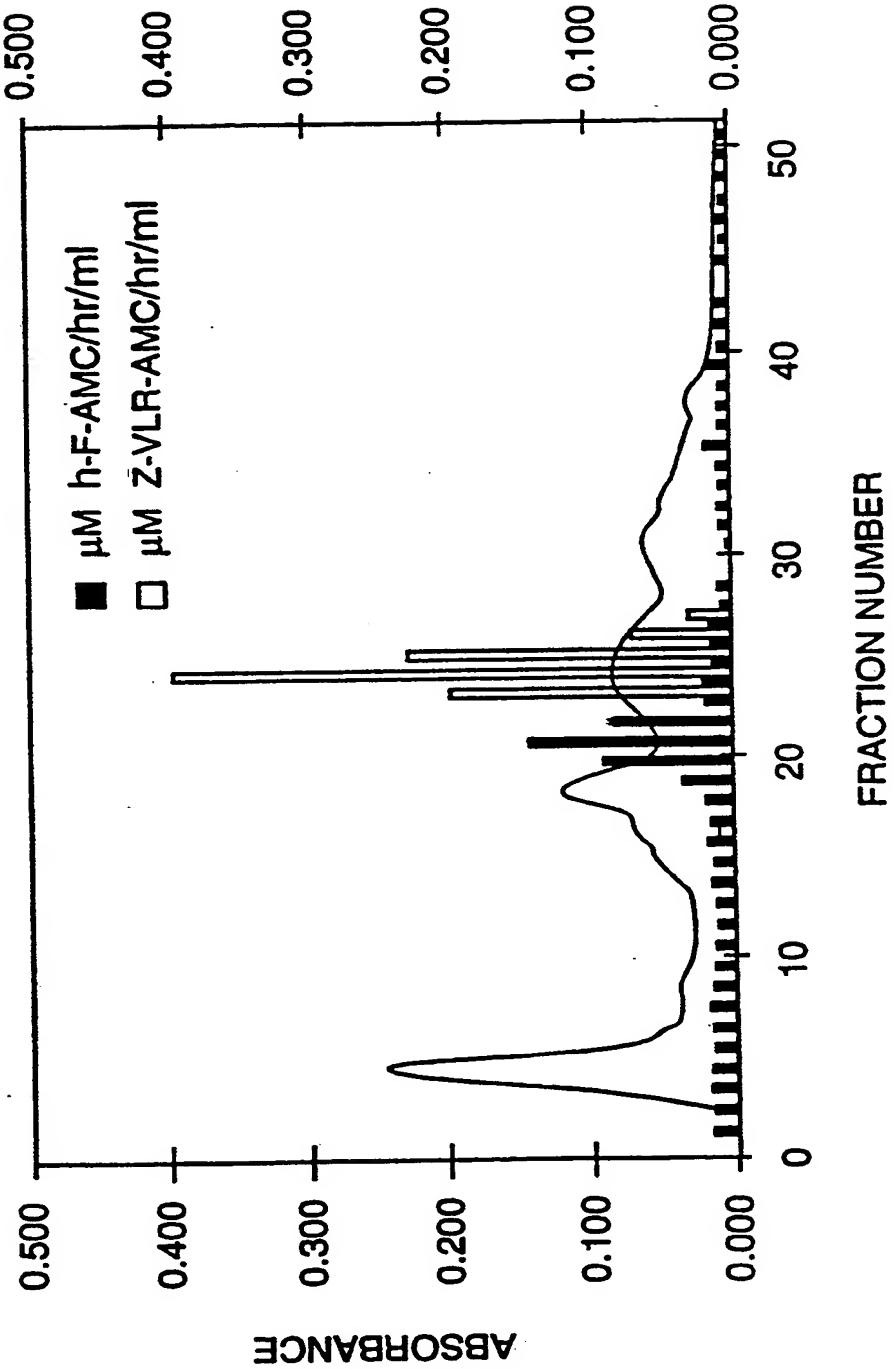

FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/09702

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 9/50, 15/00, 9/64; A61K 37/54, 39/00
US CL :424/94.63, 85.8; 435/172.3, 252.3, 219, 226

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/94.63, 85.8; 435/172.3, 252.3, 219, 226

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Dialog

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Molecular and Biochemical Parasitology, volume 33, issued 1989, Gamble et al., "Purification of a 44 Kilodalton Protease which Mediates the Ecdysis of Infective Haemonchus contortus larvae", pages 49-57, see entire document.	1,3 2, 4-17
Y	PNAS, Volume 78, Number 11, issued November 1981, Suggs et al., "Use of synthetic oligonucleotides as hybridization probes: isolation of cloned cDNA sequences for human Beta-2 microglobulin", pages 6613-6617, see entire document.	1-17
Y	Journal of Parasitology, volume 73, no. 1, issued February 1987, Tamashiro et al., "Proteolytic cleavage of IgG and other protein substrates by Dirofilaria immitis microfilarial enzymes", pages 149-154, see entire document.	1-17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 26 January 1993	Date of mailing of the international search report 10 FEB 1993
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer KEITH D. HENDRICKS Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/09702

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Biological Chemistry, vol. 260, No. 12, issued 25 June 1985, Hotez et al., "Isolation and Characterization of a Proteolytic Enzyme from the Adult Hookworm Ancylostoma caninum" pages7343-7348, see entire document.	1-17
Y	Molecular and Biochemical Parasitology, volume 35, issued 1989, Dalton et al., "Thiol proteases released in vitro by Fasciola hepatica", pages 161-166, see entire document.	1-17
Y	Epidemiologic Reviews, volume 5, issued 1983, Grieve et al., "Epidemiology of Canine Heartworm Infection", pages 220-246, see entire document.	1-17
Y	Journal of Parasitology, vol. 73, no. 2, issued April 1987, Abraham et al., "In vitro culture of Dirofilaria immitis third- and fourth- stage larvae under defined conditions", pages 377-383, see entire document.	1-17
Y	Journal of Helminthology, vol. 60, issued 1986, Maki et al., "Demonstration of carboxyl and thiol protease activities in adult Schistosoma mansoni, Dirofilaria immitis, Anjostrongylus cantonensis, and Ascaris suum", pages 31-37, see entire document.	1-17

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